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Title

Wound healing effects of *Cajanus cajan* (L.) Millsp and *Dalbergia latifolia* Roxb.

Aim:

To study the phytochemical content and the *in-vitro* wound healing effects of two plants - *Dalbergia latifolia* Roxb., and *Cajanus cajan* (L.) Millsp.

Objectives:

1. To screen the plants for phytochemicals and their content.
2. To assess antibacterial activity.
3. To assess cell proliferative activity.
4. To investigate the wound healing property *in-vitro*.

Background:

Skin plays a critical function in maintaining fluid balance, preventing infections, and controlling thermogenesis from infancy through old age. For patient and society, disruption of this regenerative protective layer could be harmful (Sinno & Prakash, 2013). A wound is an injury brought on by chemical, physical, or thermal factors that compromises the surface integrity of skin and its underlying cells/tissues (Greaves et al., 2013).

Wound healing (WH) involves the synchronized interaction between various cell types, proteins, growth factors, and extracellular matrix components (ECM) in a complex cascade of reactions to ultimately restore the normal anatomy and functioning of the tissue (Sinno & Prakash, 2013). This process generally comprises four intersecting but well-defined stages: a) Haemostasis, b) Inflammation, c) Proliferation and d) Remodelling (Guo & DiPietro, 2010; Landén et al., 2016; Visha & Karunagaran, 2019).

The first stage of haemostasis occurs when blood vessels contract and fibrin clot formation commences at the wound site shortly after an injury. Pro-inflammatory cytokines and growth factors like transforming growth factor (TGF)-, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) are also released in the tissue (Guo & DiPietro, 2010).

Once the bleeding stops, inflammatory cells enter the wound via chemotaxis, facilitating the inflammatory phase. Neutrophils, macrophages, and lymphocytes assemble in succession. Proteases and reactive oxygen species (ROS) are released by neutrophils to clear up invading bacteria and get rid of debris at the wound site (Guo & DiPietro, 2010). At an early stage,

macrophages release cytokines which promote inflammatory response by activating leukocytes. Later, the macrophages undergo phenotypic change and eliminate the neutrophils, in turn signalling the keratinocytes and fibroblasts to facilitate angiogenesis advancing the healing pathway to proliferative stage. Lymphocytes peak at early proliferative to late remodelling stage. They support growth of fibroblast and keratinocytes by producing fibroblast growth factor (FGF-7), keratinocytes growth factor (KGF) and insulin-like growth factor (IlF-1) (Guo & DiPietro, 2010).

At proliferative phase, re-epithelialization of the damaged derma begins. The fibroblast promotes growth of main components of the ECM- collagen, glycosaminoglycans and proteoglycans. They, along with epithelial cells also support the growth of capillaries and formation of granulation tissue at the wound site.

The last stage of remodelling comes after vigorous proliferation and ECM synthesis. In this stage, recently produced capillaries begin to degenerate, and the normal blood vessel density is restored. It is crucial at this stage that the ECM remodels to a structure of that of normal tissue. The myofibroblast facilitates wound contraction throughout the healing process (Guo & DiPietro, 2010). After complete healing, the resultant scar may have about 80% of the tensile strength of the original skin that it replaced (Visha & Karunagaran, 2019).

There are multiple factors which affect this normal WH cascade. External factors like availability of oxygen, temperature, the mode and cause of injury, wound size and severity, infection caused by bacteria or fungi, etc., greatly affect the time and any external aid required for complete healing of a wound. Internal factors too like age, existing comorbidities like diabetes, blood pressure, etc., stress, hormone levels, medications like steroids, etc., also have a significant impact on healing (Visha & Karunagaran, 2019).

All such factors can interfere with the normal healing process and lead to delayed healing. Depending upon the size and severity, if a wound fails to heal completely after 3-9 weeks from its initial occurrence, it is termed as a chronic wound (Kyaw et al., 2018). Chronic wounds do not undergo organized repair mechanisms required to regain structural and functional integrity (Bowers & Franco, 2020; Frykberg & Banks, 2015; Han & Ceilley, 2017). Assessment of the type of wound is done based on the location, depth, size, tissue affected, etc. Most chronic wounds can be classified by etiology as pressure ulcers, arterial ulcers, diabetic ulcers, or venous ulcers based on location and appearance (Bowers & Franco, 2020; Frykberg & Banks, 2015).

One of the major and most frequent causes of chronic wounds is infection by pathogenic bacteria or fungi (Han & Ceilley, 2017). Complexity arises when multiple bacterial species reside at the wound site and form biofilms. Communities of bacteria in biofilms are frequently bound to surfaces or to one another and are enmeshed in a matrix of extracellular polymeric molecules (EPS). The EPS encourages the biofilm's intrinsic resistance to both antimicrobials and immune responses (Percival et al., 2010). Multiple microbial species isolated from infected wounds showed resistance to majority of common antibiotics like Ampicillin, amoxicillin, ertapenem, etc (Bessa et al., 2015). *Pseudomonas aeruginosa*, *Staphylococcus aureus* were found in most infections along with *E.coli, B. subtilis, Proteus mirabilis, Corynebacterium sps* and *Micrococcus* more common than others (Bessa et al., 2015; Percival et al., 2010; Puca et al., 2021)(Howell-Jones et al., 2005). As a wound becomes chronic, the healing process gets

stuck at the inflammatory stage resulting in extreme pain and discomfort. A buildup of ROS at the wound site degrades any healthy growing cells leading to prolongation of the non-healing state. The presence of pathogenic multi-drug resistant bacteria in addition to sustained inflammation and ROS has led to a major concern in developing effective treatment methods or therapeutic approaches for wound care and management. Hence, research needs to be undertaken in finding cost-effective alternate treatment methods which can combat the drug-resistant bacterial species as well as facilitate the progression of the healing pathway in a natural systemized manner.

Ancient Egyptians, native people of Africa and Asia, Romans, and Americans have all employed medicinal herbs as the initial mode of treatment for inflammation, burns, ulcers, and surgical wounds for more than 5000 years (Budovsky et al., 2015; Sharma et al., 2021). Numerous organic bioactive substances found in plants have been shown to hasten WH and promote tissue regeneration (Thangapazham et al., 2016). The active phytochemicals are of much importance because of their therapeutic effects, employing them as treatment lowers the risk of side effects otherwise caused by antibiotics and other drugs (Budovsky et al., 2015; Thangapazham et al., 2016). In recent years, the efficacy of herbal drugs for WH have been well-researched and documented with significant results (Vitale et al., 2022). Plants like *Aloe vera*(Yazarlu et al., 2021), *Curcuma longa*(Akbik et al., 2014), *Azadirachta indica*(Alzohairy, 2016), *Sesamum indicum*(Pereira & Bártolo, 2016), etc., have shown significant results in both *in-vitro* and *in-vivo* tests.

Cajanus cajan (L.) Millsp. is a plant belonging to the legume family Fabaceae. It is one of the most common legume plant, widely cultivated and consumed as *dal* in India. It is locally called *Arhar, Tur dal*, or pigeon pea. Apart from its nutritional value and consumption, it also has a number of traditional medicinal uses. Paste made from leaves is applied on wounds(*The Useful Plants of India*, n.d.), oral ulcers and inflammation. Leaves are also used to stop bleeding in Chinese medicine, to relieve pain and to get rid of infection. People of Tamil Nadu employ seed and young stem in treatment of stomatitis and gingivitis. Leaf and seed paste are applied over breast to induce lactation. Garo tribe of Bangladesh use the leaves to cure diabetes. The leaves have shown antibacterial, antioxidant properties in invitro and *in-vivo* studies. (Pal et al., 2011).

Dalbergia latifolia Roxb. is another member of family Fabaceae which has been documented to be traditionally used to treat wounds. The root, bark and leaves of the plant have been employed for the treatment of chronic wound ulcers, leprosy and leukoderma (G.V. Satyavati et al., 1987; K. R. Kirtikar & B. D. Basu, n.d.; S.K Jain, 1991; S.M Almeida & M.R Almeida, 2010). The methanolic extract of leaves showed high antibacterial activity against multi-drug resistant *S. aureus, Proteus mirabilis and P. aeruginosa* strains clinically isolated from patients suffering from urinary tract infection, (Mishra & Padhy, 2013) as well as significant activity against several strains of clinically isolated multi-drug resistant *Salmonella enterica* (Jemiseye et al., 2018). The plant has also shown significant results in vitro anti-oxidant activity for reducing power assay, DPPH-scavenging assay and NO radical scavenging assay. Methanol extract from leaves demonstrated considerable antioxidant activity against 5-FU-induced

oxidative stress in albino rats (Deshmukh et al., 2021). A strong alpha-glycosidase inhibitory potential was found in a methanolic extract of *D. latifolia* leaves, with an IC50 value of 0.947 mg/ml (Tripathi R, 2018).

C. cajan and D. latifolia have both exhibited compelling results in *in-vitro* and *in-vivo* tests for their anti-oxidant, anti-inflammatory, anti-diabetic and anti-bacterial potential. However, their traditional use as a wound-healing agent has seldom been explored. Since plant-derived wound healing agents might prove to be an effective alternative mode of treatment, this study aims to explore the potentials of such plants which have relevant traditional use but no scientific evidence to support them, beginning with these two plants.

Significance:

Persistent chronic wounds severely affect the patient's quality of life, and impart significant financial and mental burden. The non-healing nature of these wounds require expensive treatment methods including surgeries, most often leading to limb amputations and in extreme cases even death. With the rise in cases of diabetes and obesity, more people are at risk of developing chronic wounds. By 2025, an estimated 400 million or more diabetic cases will prevail globally, with the largest growth occurring in South America, Asia, and Africa (S. Gupta et al., 2021). Yazdanpanah *et al* (2018) estimates that 15–25% of these patients will develop diabetic foot ulcers during their lifetime (Yazdanpanah et al., 2018).

In developing nations such as India, the problem of wounds is exacerbated by additional factors such as illiteracy, lack of quality healthcare, especially in rural areas; expensive equipment due to imports, financial inadequacy and a lack of universal health insurance coverage (Shukla et al., 2005). A leading doctor has stated that wound care and management has become a major concern in India as it is a neglected field and Indian doctors refuse to take up the challenge (Shahid Akhtar, 2019). As a consequence, limited data is available on Indian figures pertaining to chronic wound cases, management and challenges in treatments. Gupta *et al*. carried out the largest community-based cross-sectional survey in India between 2001 and 2003. According to the survey, there were 15.03 wounds for every 1000 people overall (N. Gupta et al., 2004). With this increasing concern, it is imperative that a solution needs to be found to prevent this from becoming a major health concern in a country which is already battling with a rising number of cancer cases, heart-related issues and diabetes to name a few.

National Status:

Several plants have traditionally been used in India to cure superficial and internal wounds. The most common among them are *Curcuma longa* (Turmeric), *Azadirachta indica* (Neem), *Aloe vera* etc. A study conducted using incision and excision wound models on swiss albino rats using hydrogel prepared from seeds of *Moringa oleifera* showed significant results as compared to the standards used (Ali et al., 2020). *Aristolochia saccata* leaf extract showed significant results on *in-vitro* migration and proliferation of L929 Human Fibroblast cell line

detected via the scratch wound assay. It was also shown to induce the expression of Collagen type-1, an ECM factor, by flow cytometry analysis (Bolla et al., 2019). Although several of these plants have been studied scientifically *in-vitro* along with *in-vivo* preclinical and clinical trials, many other plants are yet to be explored for their potential in this regard.

International Status:

Plants have also been used globally to treat wounds in traditional medicine systems like Thai(TTM) or Chinese(CTM) Traditional medicine. *Nigella sativa* seeds as a treatment for wound and inflammation in the oral cavity is found in the Mukkharoka scripture of TTM along with dry fruits of S*olanum trilobatum L.* as the main ingredient of Phra-sa-ma-vaeng recipe as in the National List of Essential Medicines of Thailand. *In-vitro* studies on these plants have significantly shown to promote cell proliferation and accelerate wound recovery (Siriwattanasatorn et al., 2020).

Study Hypothesis:

The plants show active phytoconstituents and have potential wound-healing properties.

Study Rationale:

Although the number of uses for the treatment of wounds by plant resources is many, there is a lack of research, trials and studies to investigate these documented ethnobotanical and traditional claims. Except for some popular plants like *Curcuma longa, Aloe vera,* etc., numerous other sources especially traditionally used plants among rural areas, tribes or communities remain unexplored for their potential.

Considering the wide traditional use of plants as a primary treatment for healing wounds in many countries including India, employing standardized methods are crucial to ascertain the therapeutic activity of the plants, their parts and identification of the active ingredients responsible for the said activity. A preliminary analysis is a must and fundamental in examining and exploring the range of phytochemicals and active constituents in these plants, as well as validating their acclaimed uses as wound healing agents by specific targeted *in-vitro* and *in-vivo* assay studies.

More emphasis on the management of chronic wounds and infections like diabetic ulcers, venous ulcers, etc is required and since *D. latifolia* and *C. cajan* among several others have documented uses for the same, it is plausible to undertake research and carry out analysis to scientifically establish and prove their implied medicinal use.

Research Methodology:

1. Preliminary Phytochemical Screening:
   Aqueous and Ethanolic extracts of plants according to standard procedures(Shaikh & Patil, 2020).

2. Quantification of Plant Secondary metabolites.
   a. Total Phenols by Folin's Ciocalteu's Method(Cicco et al., 2009).
   b. Total Flavonoids by Chang (2002) (Chang et al., 2020)
   c. Total Alkaloids (Sreevidya & Mehrotra, 2003).
   d. Total Cardiac Glycosides by Baljet Reaction(Shaikh & Patil, 2020).

3. Class of compound analysis by HPTLC fingerprinting.

4. Antibacterial assay.
   Test Organisms- Gram +ve: *Staphylococcus aureus, Bacillus subtilis and Corynebacterium sp.*
   Gram -ve: *Pseudomonas. aeruginosa, Proteus mirabilis and Escherichia coli.*
   Methods: a) Disc diffusion method(Bauer et al., 1966): To calculate Zone of Inhibition(ZI).
   b) Broth dilution method(Wiegand et al., 2008): To calculate Minimum Inhibitory Concentration(MIC).
   b) TLC Autobiography(Balouiri et al., 2016): to identify the active antibacterial phytoconstituents.

5. Cytotoxicity assay of plants and Cell regeneration/Proliferation efficacy of whole extract and TLC fractions on Human Epidermal Fibroblast Cell line (HDF):
   a) LDH assay (Kumar et al., 2018) for Cytotoxicity testing.
   b) Cell proliferative testing by MTT assay(Mosmann, 1983) ,WST-1 assay(Nissen et al., 1997) and ATP-bioluminescent assay(Crouch et al., 1993).

6. Best extracts from Antibacterial and Cell Proliferative assay to be selected and Scratch wound assay to be performed (Chun-Chi Liang *et al*)(Liang et al., 2007) on HDF cell line.

Statistical Plan:

1. The phytochemical screening and quantitative assays are to be done in triplicates and results expressed as means.
2. Antibacterial efficacy to be assessed as MIC and results expressed as Mean ± SEM.
3. Dose dependency of the extracts on in vitro assay results to be analyzed by Coefficient of Correlation.

4. All the in vitro assay test results of individual fractions and whole extracts are to be compared using one-way ANOVA with Tukey's Honestly-Significant Difference to check the significance of differences at p=0.05.

Limitations of the Study:

Even though in vitro test on Human cell lines will suffice to ascertain the wound healing potential of plants to be studied, future progress for clinical trials may require a pre-clinical test on animal models.

Expected Outcomes of Study:

1. Phytochemical analysis of plants will give insights into the bio-active constituents present in the plants responsible for the therapeutic effects.
2. Antibacterial assays will establish the potential of the plants to be used as an antibacterial agent against specific bacterial strains.
3. The cell proliferative assay will give an understanding of the ability of plant extracts to facilitate the multiplication of cells.
4. Scratch- wound assay will demonstrate the plant's wound healing property.
5. Positive results for tested activities will scientifically validate the traditional claims.

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